(1) The investigation may be terminated or suspended in its entirety until the investigator is reinstated under §54.219 or another investigator accepts responsibility for the investigation.

(2) No new subject shall be allowed to participate or be requested to participate in the investigation until the investigator is reinstated under §54.219 or another investigator accepts responsibility for the investigation.

(3) Any human subject who has previously been allowed to participate in the investigation and who remains under the supervision of the investigator, but who is no longer receiving the test article or having it used involving him or her, i.e., one having follow-up monitoring by the investigator or one action as a control, shall continue to be monitored by the investigator but shall not again receive the test article, or have it used involving him or her, until the investigator is reinstated under §54.219 or another investigator accepts responsibility for the investigation.

(4) Any human subject who has been allowed to participate in the investigation and who, but for suspension of the investigation would continue to receive the test article or have it used involving him or her, shall not receive it or have it used until either:

(i) Another investigator accepts responsibility for the investigation; or

(ii) The disqualified investigator determines in writing that it is contrary to the health of the subject to defer further use of the test article until another investigator can assume responsibility for the investigation. In such a case, the Commissioner may impose any further conditions that the Commissioner deems appropriate to protect the rights and safety of the subjects.

(c) Once an investigator has been disqualified, each application for a research or marketing permit, whether approved or not, containing or relying upon any clinical investigation performed by the investigator may be examined to determine whether the investigation was or would be

essential to a regulatory decision regarding the application. If it is determined that the investigation was or would be essential, the Commissioner shall also determine whether the investigation is acceptable, notwithstanding the disqualification of the investigator. Any investigation done by an investigator before or after disqualification may be presumed to be unacceptable, and the person relying on the investigation may be required to establish that the investigation was not affected by the circumstances which led to disqualification of the investigator, e.g., by submitting validating information. If the investigation is determined to be unacceptable, such investigation shall be eliminated from consideration in support of the application, and such elimination may serve as new information justifying the termination or withdrawal of approval of the application.

(d) No clinical investigation begun by an investigator after the date of his or her disqualification shall be considered in support of any application for a research or marketing permit, unless the investigator has been reinstated under §54.219. The determination that a clinical investigation may not be considered in support of an application for a research or marketing permit does not, however, relieve the applicant for such a permit of any obligation under any other applicable statute ore regulation to submit the results of the investigation to the Food and Drug Administration.

§54.213 Public disclosure of information regarding disqualification.

(a) Upon issuance of a final order disqualifying an investigator, the Commissioner may notify all or any interested persons. Such notice may be given in the discretion of the Commissioner whenever the Commissioner believes that such notice would further the public interest or would promote compliance with the regulations set forth in this part. Such notice, if given, shall include a copy of the final order issued under §54.206(a) and shall state that the disqualification constitutes a determination by the Commissioner that the

investigator is not eligible to conduct clinical investigations subject to requirements for prior submission to the Food and Drug Administration and that the results of any clinical investigations conducted by the investigator may not be considered by the Food and Drug Administration in support of any application for a research or marketing permit. The notice shall further state that it is given because of the professional relations between the investigator and the person notified and that the Food and Drug Administration is not advising or recommending that any action be taken by the person notified.

(b) A determination that an investigator has been disqualified and the administrative record regarding such determination are disclosable to the public under part 20 of this chapter.

(c) Whenever the Commissioner has reason to believe that an investigator may be subject to disqualification, the Commissioner may, in the Commissioner's discretion, so notify the sponsor of any ongoing clinical investigation in which that investigator is participating simultaneously with or subsequent to proposing disqualification of the investigator under §54.204(a), unless there are overriding safety considerations that warrant earlier notification of the sponsor.

§54.215 Alternative or additional actions to disqualification.

Disqualification of an investigator under this subpart is independent of, and neither in lieu of nor a precondition to, other proceedings or actions authorized by the act. The Commissioner may at any time, through the Department of Justice, institute any appropriate judicial proceeding (civil or criminal) and any other appropriate regulatory action, in addition to or in lieu of, and before, at the time of, or after disqualification. The Commissioner may also refer pertinent matters to another Federal, State, or local government agency for such action as that agency determines to be appropriate.

§54.217 Suspension or termination of an investigator by a sponsor.

The sponsor of a clinical investigation may at any time remove an investigator from further participation in the investigation, whether or not the Commissioner has commenced any action to disqualify the investigator. The sponsor need not utilize either grounds or the procedures for disqualification set forth in this subpart. If a sponsor removes an investigator from a clinical investigation, the sponsor shall notify the appropriate Bureau within the Food and Drug Administration in writing of the reasons for such removal as soon as possible, but in no event later than 15 working days after such removal.

§54.219 Reinstatement of a disqualified clinical investigator.

(a) An investigator who has ben disqualified may be reinstated as eligible to conduct clinical investigations subject to requirements for prior submission to the Food and Drug Administration, or as acceptable to be the source of clinical investigations to be submitted to the Food and Drug Administration, if the Commissioner determines, upon an evaluation of a written submission from the investigator, that the investigator can adequately assure that he or she will conduct such studies in compliance with the requirements set forth in this part and other applicable regulations in this chapter, e.g., parts 312, 511, or 812.

(b) A disqualified investigator who wishes to be so reinstated shall present in writing to the Commissioner reasons why he or she believes he or she should be reinstated and a detailed description of the corrective actions the investigator has taken or intends to take to assure that the acts or omissions that led to disqualification will not recur. The Commissioner may condition reinstatement upon the submission or special undertakings by a sponsor, an institution, an institutional review board, or another investigator to review in detail the investigator's compliance with the applicable regulations upon an inspection.

APPENDIX B

ORGANIZATION FOR ECONOMIC COOPERATION AND DEVELOPMENT (OECD) GUIDELINES FOR *IN VITRO* GENOTOXICITY STUDIES

STUDY PROTOCOL - 1

SALMONELLA - ESCHERICHIA COLI/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY WITH A CONFIRMATORY TRIAL

I. OBJECTIVE

The objective of this study is to evaluate the test article for its ability to induce reverse mutations at: i) the histidine locus in the genome of several strains of *Salmonella typhimurium*; and at ii) the tryptophan locus of *Escherichia coli* strain WP2*uvr*A.

II. TEST SYSTEMS

Salmonella typhimurium: The *Salmonella*/mammalian-microsome reverse mutation assay detects point mutations, both frameshifts and/or base pair substitutions. The strains of *Salmonella typhimurium* used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in their histidine operon. When these histidine-dependent cells (*his*-) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of histidine) only those cells which revert to histidine independence (*his*+) are able to form colonies. The trace amount of histidine in the media allows all the plated bacteria to undergo a few cell divisions, which is essential for mutagenesis to be fully expressed. The *his*+ revertants are readily discernable as colonies against the limited background growth of the *his*- cells. By utilizing several different tester strains, both base pair substitution mutations and frameshift mutations can be detected. The Ames Test has been shown to be a sensitive, rapid, and accurate indicator of the mutagenesis induced by both chemical and physical treatments.

Escherichia coli: The *Escherichia coli* WP2*uvr*A reverse mutation assay detects point mutations, specifically base pair substitutions. The *Escherichia coli* tester strain WP2*uvr*A used in this assay is a tryptophan auxotroph (*trp*$^-$) by virtue of a conditionally lethal mutation at a site which blocks a step of tryptophan biosynthesis prior to the formation of anthranilic acid. Since the target site for true back mutation is an ochre nonsense mutation, tryptophan-independent revertants (*trp*$^+$) can arise either by a base change at the site of the original alteration or by suppression by specific suppressor mutations at a second site in tRNA genes (Brusick et al., 1980). When the tryptophan-dependent cells (*trp*-) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of tryptophan) only those cells which revert to tryptophan independence (*trp*+) are able to form colonies. The trace amount of tryptophan in the media allows all the plated bacteria to undergo a few cell divisions, which is essential for mutagenesis to be fully expressed. The *trp*+ revertants are readily discernable as colonies against the limited background growth of the *trp*- cells.

III. MATERIALS

A. Tester Strains

Salmonella typhimurium: the tester strains to be used will be the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, and TA1537 as described by Ames et al. (1975). The specific genotypes of these strains are shown in Table 1.

TABLE 1 SALMONELLA TESTER STRAIN GENOTYPES

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R Factor
TA1535	TA1537		*rfa*	*uvr*B	-
TA100		TA98	*rfa*	*uvr*B	+R

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e., benzo[a]pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the *uvr*B gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA1535 is reverted by base substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.
Escherichia coli: the tester strain to be used will be the tryptophan auxotroph WP2*uvr*A as described by Green and Muriel (1976).

In addition to a mutation in the tryptophan operon, the tester strain contains a *uvr*A DNA repair deficiency which enhances its sensitivity to some mutagenic agents. This deficiency allows the strain to show enhanced mutability if the *uvr*A repair system would normally act to remove the damaged part of the DNA molecule and accurately repair it afterwards.

Tester strain WP2*uvr*A is reverted from tryptophan dependence (auxotrophy) to tryptophan independence (prototrophy) by base substitution mutagens.

Source:

a. *Salmonella typhimurium*: the tester strains will be obtained directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

b. *Escherichia coli*: tester strain WP2*uvr*A will be obtained from a suitable repository.

Storage:

a. Frozen Permanent Stocks
Frozen permanent stocks will be prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing away appropriately vialed aliquots. Frozen permanent stocks of the tester strains will be stored at ≤-70°C.

b. Master Plates
Master plates will be prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with either histidine and biotin (and for strains containing the R-factor, ampicillin) or tryptophan. Tester strain master plates will be stored at 5 ± 3°C.

Preparation of Overnight Cultures:

a. Inoculation
Overnight cultures for use in all testing procedures, will be inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks will be placed in a shaker/incubator which will be programmed to begin operation (shaking, 125 ± 25 rpm; incubation, 37 ± 2°C) so that the overnight cultures are in log phase or late log phase when turbidity monitoring begins.

b. Harvest
To ensure that cultures are harvested in late log phase, the length of incubation will be determined by spectrophotometric monitoring of culture turbidity. Cultures will be harvested once a predetermined turbidity is reached as determined by a percent transmittance (%T) reading on a spectrophotometer. This target turbidity ensures that cultures have reached a density of at least 0.5×10^9 cells per ml and that the cultures have not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures will be removed from incubation when the target %T is reached and placed at 5 ± 3°C.

Confirmation of Tester Strain Genotypes:

a. *Salmonella typhimurium*: tester strain cultures will be checked for the following genetic markers on the day of their use in the mutagenicity assay:

   i) *rfa* Wall Mutation
   The presence of the *rfa* wall mutation will be confirmed by demonstration of the cultures sensitivity to crystal violet.

   ii) pKM101 Plasmid
   The presence of the pKM101 plasmid will be confirmed for cultures of tester strains TA98 and TA100 by demonstration of resistance to Ampicillin.

   iii) Spontaneous Reversion
   The number of spontaneous revertants per plate in the vehicle controls that is characteristic of the respective strains will be demonstrated by plating aliquots of each culture along with the appropriate vehicle on selective medium.

b. *Escherichia coli*: tester strain WP2*uvr*A will be checked for the following genetic marker on the day of its use in the mutagenicity assay:

   i) Spontaneous Reversion
   The number of spontaneous revertants per plate in the vehicle controls that is characteristic of WP2*uvr*A will be demonstrated by plating aliquots of each culture along with the appropriate vehicle on selective medium.

Tester Strain Media:

a. Culturing Broth
The broth used to grow overnight cultures of the tester strains will be Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (W/V) Oxoid Nutrient Broth #2 (dry powder).

b. Minimal Bottom Agar Plates
Bottom agar (25 ml per 15 x 100 mm petri dish) will be Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) supplemented with 0.2% (W/V) glucose.

c. Top Agar for Selection of Revertants
Top (overlay) agar will be prepared with 0.7% agar (W/V) and 0.5% NaCl (W/V) and will be supplemented with 10 ml of 1) 0.5 mM histidine/biotin solution per 100 ml agar for selection of histidine revertants, or 2) 0.5 mM tryptophan solution per 100 ml agar for the selection of tryptophan revertants.

B. Controls

1. Negative Controls
Negative controls will be plated for all strains.

2. Positive Controls
The combinations of positive controls and tester strains plated concurrently with the assay are indicated in Table 2.

TABLE 2 POSITIVE CONTROLS

Tester Strain	Positive Control	Conc. Per Plate
TA98	2-nitrofluorene	1.0 µg
TA100	sodium azide	2.0 µg
TA1535	sodium azide	2.0 µg
TA1537	ICR-191	2.0 µg
WP2*uvr*A	4-nitroquinoline-N-oxide	10.0 µg

3. Test Article
The test article will be radio frequency radiation at 837 MHz generated as specified by the sponsor.

IV. METHODS

A. Dose Range Finding Study

The growth inhibitory effect (cytotoxicity) of the test article to the test system will be determined in order to allow the selection of appropriate doses to be tested in the mutagenicity assay.

Design: The dose range finding study will be performed using tester strains TA100 and WP2*uvr*A. A minimum of three treatments with test article will be tested at one plate per treatment. The test article will be checked for cytotoxicity up to the maximum exposure level specified by the sponsor.

Rationale: The growth inhibitory effect (cytotoxicity) of the test article on tester strain TA100 is generally representative of that observed on the other *Salmonella typhimurium* tester strains. Because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. The *Escherichia coli* tester strain WP2*uvr*A does not possess the *rfa* wall mutation that the *Salmonella typhimurium* strains have, and thus, a different range of cytotoxicity may be observed.

Evaluation of the Dose Range Finding Study: Cytotoxicity is detectable as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn.

Selection of the Maximum Dose for the Mutagenicity Assay:

Cytotoxicity Observed: When cytotoxicity is observed in the dose range finding study, the highest exposure level to be used in the subsequent mutagenicity assay will be that which gives a detectable reduction of revertants per plate and/or a thinning or disappearance of the bacterial background lawn.
No Cytotoxicity Observed: If no cytotoxicity is observed in the range finding study, then the highest exposure level to be used in the mutagenicity assay will be the maximum exposure specified by the sponsor.

B. Mutagenicity Assay

Design: The assay will be performed using tester strains TA98, TA100, TA1535, TA1537, and WP2*uvr*A. If cytotoxicity has been demonstrated in the range finding study, a minimum of three treatments of test article will be tested along with the appropriate negative and positive controls. If no cytotoxicity has been demonstrated, a minimum of three treatments will be tested.

Once the results of the mutagenicity assay have been reviewed, a confirmatory assay will be performed. Routinely, the same exposure levels will be used in the confirmatory assay as were used in the initial mutagenicity assay. However, the Study Director may modify the exposures to be used in the confirmatory assay for such reasons as cytotoxicity or clarification of equivocal responses.

Frequency and Route of Administration: The tester strains will be exposed to the test article via the plate incorporation methodology originally described by Ames et al. (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of mutagens. The exposed bacteria will be suspended in molten agar which is overlaid onto a minimal agar plate. Following incubation at 37 ± 2°C for 48 ± 8 hr, revertant colonies will be counted. All treatments with test article, negative controls, and positive controls will be plated in triplicate.

C. Plating Procedures

These procedures will be used in both the dose range finding study and the mutagenicity assay. Each plate will be labeled with a code which identifies the test article, test phase, tester strain, and exposure level.

After the exposure period, 100 µl of tester strain will be added to 2.5 ml of molten selective top agar (maintained at 45 ± 2°C). The mixture will be vortexed and overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay has solidified, the plates will be inverted and incubated for 48 ± 8 hr at 37 ± 2°C. Positive control articles will be plated using a 50 µl plating aliquot.

D. Scoring the Plates

Plates which are not evaluated immediately following the incubation period will be held at 5 ± 3 °C until such time that colony counting and bacterial background lawn evaluation can take place.

1. Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn will be evaluated for evidence of cytotoxicity. Evidence of cytotoxicity will be scored relative to the negative control and recorded along with the revertant counts for that dose.

2. Counting Revertant Colonies

Revertant colonies for each tester strain within a given test article treatment series will be counted either entirely by automated colony counter or entirely by hand (with the exception of the range finding study).

E. Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation will be calculated.

V. EVALUATION OF TEST RESULTS

Before assay data can be evaluated, the criteria for a valid assay must be met.

A. Criteria For a Valid Assay

The following criteria will be used to determine a valid assay:

1. Tester Strain Integrity: *Salmonella typhimurium*

a. *rfa* Wall Mutation: To demonstrate the presence of the *rfa* wall mutation, tester strain cultures must exhibit sensitivity to crystal violet.

b. pKM101 Plasmid: To demonstrate the presence of the R-factor plasmid, pKM101, cultures of tester strains TA98 and TA100 must exhibit resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants: To demonstrate the requirement for histidine, the tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable ranges for the vehicle controls are as follows:

TA98	8 - 60
TA100	60 - 240
TA1535	4 - 45
TA1537	2 - 25

2. Tester Strain Integrity: *Escherichia coli*

Characteristic Number of Spontaneous Revertants: To demonstrate the requirement for tryptophan, the tester strain culture must exhibit a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable range for the WP2*uvr*A vehicle controls is 5 to 40 revertants per plate.

3. Tester Strain Culture Density:

To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures must be greater than or equal to 0.5×10^9 bacteria per ml and/or have reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per ml.

4. Positive Control Values

To demonstrate that the tester strains are capable of identifying a mutagen, the mean value of a positive control for a respective tester strain must exhibit at least a three-fold increase over the mean value of the negative control for that strain.

5. Cytotoxicity

a. A minimum of three non-toxic dose levels will be required to evaluate assay data.

B. Criteria For A Positive Response

Once the criteria for a valid assay have been met, responses observed in the assay are evaluated as follows:

1. Tester Strains TA98, TA100 and WP2uvrA
For a test article to be considered positive, it must produce at least a two-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate negative control. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing exposure levels of the test article.

2. Tester Strains TA1535 and TA1537
For a test article to be considered positive, it must produce at least a three-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate negative control. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing exposure levels of the test article.

VI. REPORTING THE RESULTS

A report of the results of this study will accurately describe all methods used for the generation and analysis of the data.

Results presented in the report for this assay will include:

- the results of the treatment range finding study (if applicable) including the number of revertants per plate and a bacterial background lawn evaluation for each exposure level
- the identity of the bacterial tester strains used in the assay
- dose levels at which the test article was tested
- individual plate counts for all treated, positive, and negative control plates
- calculated mean and standard deviation for all replicate plate counts
- evaluation of results

VII. CHANGES AND REVISIONS

Any changes or revisions of this approved protocol will be documented, signed by the Study Director, dated, and maintained with this protocol. The Sponsor will be notified of any changes or revisions.

VIII. RECORDS TO BE MAINTAINED

All raw data, documentation, records, the protocol, and the final report generated as a result of this study will be archived.

IX. REFERENCES

Ames BN, McCann J,Yamasaki E. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. 1975. Mutat Res 31:347-364.

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Green MHL and Muriel WJ. Mutagen testing using *trp*$^+$ reversion in *Escherichia coli*. Mutat Res 1976;38:3-32.

Maron DM and Ames B. Revised Methods for the *Salmonella* Mutagenicity Test. Mutat Res 1983;113:173-215.

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STUDY PROTOCOL - 2

L5178Y TK+/- MOUSE LYMPHOMA FORWARD MUTATION ASSAY WITH A CONFIRMATORY TRIAL

I. OBJECTIVE

The objective of the assay is to determine the ability of a test article to induce forward mutation at the thymidine kinase locus as assayed by colony growth of L5178Y mouse lymphoma cells in the presence of 5-trifluorothymidine (TFT).

II. RATIONALE

Thymidine kinase (TK) is an enzyme that allows cells to salvage thymidine from the surrounding medium for DNA synthesis. If the thymidine analog TFT is included in the growth medium, the analog will be phosphorylated via the TK pathway and will cause cellular death by inhibiting DNA synthesis. Cells which are heterozygous at the TK locus (TK+/-) may undergo a single-step forward mutation to the TK-/- genotype in which little or no TK activity remains. Such mutants are as viable as the heterozygotes in normal medium because DNA synthesis proceeds by *de novo* synthetic pathways that do not involve thymidine as an intermediate. TK-/- mutants cannot utilize toxic analogs of thymidine. Cells which grow to form colonies in the presence of TFT are therefore assumed to have mutated, either spontaneously or by the test article, at the TK-/- locus.

III. MATERIALS

A. Indicator Cells

The mouse lymphoma L5178Y cell line, heterozygous at the TK locus and designated as clone 3.7.2C, will be used for this assay. All laboratory cultures will be maintained in logarithmic growth by serial subculturing for up to 4 months and will then be replaced by cells from the frozen stock. Cultures will be grown in a shaker incubator at approximately 37°C. A continuous cell stock log will be kept to record growth, doubling times (approximately 10 hours), and subculture operations. Laboratory cultures will be periodically tested for mycoplasma contamination and karyotype. To reduce the frequency of spontaneous TK-/- mutants prior to use in a mutation assay, cell cultures will be exposed to conditions which select against the TK-/- phenotype, then returned to normal growth medium for 3 to 8 days.

B. Media

The medium used for this study will be RPMI 1640 (Amacher et al., 1980; Clive et al., 1987) supplemented with horse serum (10% by volume), Pluronic® F68, L-glutamine, sodium pyruvate, and antibiotics. Treatment media will be Fischers media with the same media supplements used in the culture media except that the horse serum concentration will be reduced to 5% by volume. Cloning medium consists of the preceding growth medium with up to 20% horse serum, without Pluronic® F68 and with the addition of agar to achieve a semisolid state. Selection medium will be cloning medium containing 3 µg/ml of TFT (Clive et al., 1987).

C. Control Articles

1. Negative control article

The negative control article will be the growth medium used in the assay. At least three negative control cultures will be included in each mutation assay.

2. Positive control articles

Mandatory reference substances for use as positive control articles are not available (Clive et al., 1987). The positive control article listed below is chosen because of the large data base available and because the chemical induces both small and large colonies (Young et al., 1991). Methyl methanesulfonate (MMS) will be used at concentrations of 10.0 and 15.0 nl/ml as a positive control.

3. Test Article

The test article will be radiofrequency radiation at 837 MHz generated as specified by the Sponsor.

IV. EXPERIMENTAL DESIGN

A. Test Article

The maximum exposure will be determined by the Sponsor, taking into account any relevant cytotoxicity information available. All control and exposed cultures will be labeled according to Standard Operating Procedures to indicate test article, trial, activation, exposure level, plate number within an exposure level, and phase of the study (i.e. selection plating efficiency or mutant selection).

B. Range Finding Cytotoxicity Assay

A preliminary range-finding cytotoxicity experiment will be performed. A range of exposures will be tested for cytotoxicity, starting with the maximum exposure selected by the Sponsor and followed by four lower exposure levels in two-fold reduction steps. The cells will be treated for an exposure time of about four hours in 10 ml of treatment medium at approximately $37^{\circ} \pm 1.5^{\circ}C$ in an orbital shaker at 80 ± 10 orbits per minute after which the cells will be centrifuged and resuspended in 20 ml of growth medium. The cells will then be incubated for approximately 24 hours using procedures identical to those for mutation experiments. A cell count will be determined after the growth period to measure reduction in cell growth relative to the concurrent vehicle control cell cultures. Four or five exposure levels will then be selected for initiation of the mutation experiments, using the following criteria:

- Exposures will be chosen to cover a toxicity range from little or no survival to no apparent effect on growth compared to the negative control, or

- If little or no toxicity is observed, the mutation experiment will be initiated with the maximum exposure level selected by the Sponsor.

Although four or more exposure levels may be selected to initiate a mutation experiment, the objective will be to carry at least three doses through the entire experiment. This procedure compensates for normal variations in cellular toxicity and helps to ensure the choice of at least three doses appropriately spaced in a relative growth range that meets the appropriate criterion above.

C. Mutation Assay

The assay procedure used is based on that reported by Clive and Spector (1975), Clive et al. (1979; 1987), and Amacher et al. (1980). The cells will be obtained from logarithmically growing laboratory stock cultures and will be seeded into a series of tubes at 6×10^6 cells per tube. The cells will be pelleted by centrifugation, the culture medium removed, and the cells resuspended in a final volume of 10 ml of treatment medium. The dosed tubes will be closed, vortexed, and placed in an orbital shaker incubator at approximately 37°C at 80 ± 10 orbits per minute. After an exposure period of about four hours, the cells will be centrifuged and resuspended in 20 ml of growth medium and returned to the orbital shaker incubator as closed-tube cultures.

A standard expression period of 2 days will be used to allow recovery, growth and expression of the TK-/- phenotype. Cell densities will be determined on Day 1 (about 24 hours after treatment) and each culture will be adjusted to 3×10^5 cells/ml in 20 ml of growth medium. If the cells in a culture fail to multiply to a density of 4×10^5/ml on the first day after treatment, the culture will be returned to the incubator without being subcultured. On Day 2, cell counts again will be determined, and appropriate cultures will be selected for cloning and mutant selection.

At least three treatments will be selected for mutant analysis. If possible, treatments are selected to include a wide range of toxic action from nontoxic to highly toxic treatment conditions (approximately 10% to 20% relative total growth). Cultures with cell densities less than approximately 3×10^5 cells/ml on Day 2 will not be considered for analysis.

A total sample size of 3×10^6 cells from each selected tube will be suspended in selection medium to recover mutants. This sample will be distributed into three 100 mm dishes such that each dish will contain approximately 1×10^6 cells. The cloning efficiency will be determined by serially diluting the sample and seeding each of three 60 mm dishes with approximately 200 cells in cloning medium. All dishes will be placed in an incubator at approximately 37°C with approximately 5% CO_2/95% humidified air. After 10 to 14 days in the incubator, colonies will be counted on an automated counter capable of size discrimination among the colonies. The smallest detectable colony should be ≤ 0.3 mm in diameter, depending on its position in the agar matrix.

The mutant frequency is calculated as the ratio of the total number of mutant colonies found in each set of three mutant selection dishes to the total number of cells seeded, adjusted by the absolute selection cloning efficiency. If one dish in either set is lost due to contamination or other cause, the colony count of the missing dish is determined by a proportion equation based upon the weights of the three dishes of the set and the colony counts in the two acceptable dishes. If a lost plate is not

available for weighing, the colony count of the lost plate is determined from the average of the two remaining acceptable plates. A mutant frequency calculated by either method will be identified by footnote in the data tables as a reminder of the reduced sample size in the event of a spurious variation.

A measurement of the toxicity of each treatment will be the relative suspension growth of the cells over the two-day expression period multiplied by the relative cloning efficiency at the time of selection. Although not strictly a measure of cell survival, this parameter (called percent relative growth) provides a measure of effectiveness of treatment and will be used as the basis for selecting doses for any necessary repeat trials.

If any of the exposure levels are identified as mutagenic, the selection plates from representative control and representative mutagenic treatments will be recounted to permit mutant colony sizing. Each selection plate will be counted as described by Hozier et al., 1985. The results will be analyzed and presented in the report.

A second trial of the mutation assay will be performed after completion and analysis of the results of the first mutation assay. The assay procedures will be identical to those used in the first assay. The exposure levels and culture number may be modified for the second trial based upon results from the first trial.

V. DATA

A. Data Presentation

The data will be used to calculate several assay parameters. The reported raw data, analyzed data and the methods of calculation are listed below:

- Test article identification, selection agent and concentration, and study dates.
- Daily cell densities in the individual cultures carried through the expression period and cloning.
- Suspension growth for negative and positive controls, calculated as (Day 1 cell density/3 x 10^5) x (Day 2 cell density/3 x 10^5 or Day 1 density if not split back).
- Relative suspension growth for test article-treated cultures. These values will be expressed as percentages of the average negative control suspension growth.
- Total mutant colonies—total number of mutant colony counts obtained from 3 x 10^6 cells sampled from one culture, seeded into selective medium, and divided among three culture dishes.
- Total viable colonies—total number of colony counts obtained from 600 cells sampled from one culture, seeded into nonselective medium, and divided among three culture dishes.
- Cloning efficiency of negative and positive controls, calculated as (total number of viable

- Relative cloning efficiency of test article-treated cultures. These values will be expressed as percentages of the average negative control cloning efficiency.

- Percent relative growth—an expression of treatment toxicity obtained by multiplying the relative suspension growth by the relative cloning efficiency/100. Negative controls are by definition set equal to 100%.

- Mutant frequency for each culture. The ratio of cells seeded for mutant selection to cells seeded for cloning efficiency is 0.5×10^4. Therefore, the mutant frequency is: (total mutant colonies/total viable colonies) $\times 2 \times 10^{-4}$. Mutant frequency will be given in units of 10^{-6}.

B. Assay Acceptance Criteria

An assay normally will be considered acceptable for evaluation of test results only if all of the following criteria are satisfied:

- The average absolute cloning efficiency of the negative controls should be between 60% and 130%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and variations in cell division during unavoidable delays between counting and cloning of many cultures. Cloning efficiencies below 60% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50% to 60% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range will be conditionally acceptable and dependent on scientific judgement of the Study Director. All assays below 50% cloning efficiency will be unacceptable.

- A minimum acceptable value for the average suspension growth of the negative controls for two days is an eight-fold increase over the original cell numbers. Lower values will render an assay unacceptable because of poor cell growth.

- The background mutant frequency (average of negative control values) for assays performed with different cell stocks is 30×10^{-6} to 120×10^{-6}. Assays with backgrounds outside this range are not necessarily invalid but will be used with caution.

- A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. The minimum acceptable mutant frequency induced by MMS is 200×10^{-6}. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria.

- For test articles causing little or no mutagenic activity, an assay should include exposure levels that reduce the relative growth to 10% or 20% of the average negative control or reach the maximum exposure level specified by the Sponsor. Relative growth represents a calculation of survival that is based on both relative suspension growth during the expression period and relative cloning efficiency at the time of plating. Because mutant frequencies increase as a function of lethality, an attempt to obtain treatments in the range of 10% to 20% relative growth must be made in order to consider the assay as conclusive. This requirement is waived if the highest assayed exposure level is at least 75% of an

excessively toxic treatment. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

- An experimental mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 60. These limits avoid problems with the statistical distribution of scorable colonies among dishes.

- Mutant frequencies for at least four treated cultures are normally determined in each assay, although a minimum of three analyzed cultures is considered necessary to accept a single assay for evaluation of the test article.

- Mutant frequencies are normally derived from sets of three dishes for both mutant colony count and viable colony count. In order to allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of two dishes per set.

C. Evaluation Criteria

Test articles are evaluated on the basis of a combination of a minimum increase in mutant frequency and a series of assay evaluation criteria. These criteria take into account the variability that can occur in an assay. An assay may need to be repeated with different exposure levels to properly evaluate a test article. The Study Director will review each assay and make recommendations as to the need for additional testing.

The minimum condition considered necessary to indicate mutagenesis for any given treatment will be a mutant frequency that is greater than or equal to two times the concurrent background frequency. Background frequency is defined as the average mutant frequency of the negative control cultures.

A mutant frequency that meets the minimum criterion for a single treated culture is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion:

- A dose-related or toxicity-related increase in mutant frequency should be observed. However, this goal depends on the exposure steps chosen for the assay and the toxicity at which mutagenic activity appears.

- A mutagenic dose-response in one mutation assay should be confirmed in the second mutation assay. While it is desirable to confirm significant mutagenesis in both trials, it may not always be possible due to normal assay variation, especially under toxic treatment conditions, and also due to the possible use of different treatment levels in the two trials.

- If the mutant frequency obtained for a single treatment at or near the highest testable toxicity is about two or more times the minimum criterion, that trial will be considered positive. However, for the test article to be evaluated as positive, the increase must be repeatable in the second trial. Lack of confirmation in the independent trial will result in a negative evaluation of the test article under the conditions of testing.

- Treatments that reduce relative total growth to less than 10% may be included in the assay but will not be used as sufficient evidence for mutagenicity as it relates to risk assessment.

A test article will be evaluated as nonmutagenic in two assays only if the minimum increase in mutant frequency is not observed for: 1) a range of exposure levels that extend to toxicity causing 10% to 20% relative growth or 2) in the case of relatively nontoxic treatments, the exposure levels extend to the maximum level selected by the Sponsor.

This presentation may not encompass all test situations, and the Study Director may use other criteria to arrive at a conclusion, especially when data from several repeat assays are available.

VI. REFERENCES

Amacher DE, Paillet SC, Turner GN, Ray VA, Salsburg DS. Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells II. Test validation and interpretation. Mutat Res 1980;72:447-474.

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Clive D, Caspery W, Kirby PE, Krehl R, Moore M, Mayo J, Oberly TJ. Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity. Mutat Res 1987;189:143-156.

Hozier J, Sawyer J, Clive D, Moore MM. Chromosome 11 aberrations in small colony L5178Y TK-/- mutants early in their clonal history. Mutat Res 1985;147:237-242.

Young R, Oveisitork F, Harrington-Brock K, Schalkowsky S, Moore M, Myhr B. Quantitative size analysis of L5178Y $TK^{-/-}$ mutant colonies in soft agar; an interlaboratory comparison. Environ Molec Mutagenesis 1991;17(Suppl 19): 79.

VII. REPORT FORMAT

The final study report will provide the following information:

- Sponsor identification
- Quality Assurance statement
- Statement of GLP compliance
- Identity and signatures of Senior Laboratory Technician and Study Director
- Test article identification
- Type of assay and protocol number
- Dates of study initiation and completion
- Methods
- Evaluation criteria
- Interpretation of results
- Conclusions

- Test results presented in tabular form
- References
- Historical control data

VIII. CHANGES OR REVISIONS

Any changes or revisions of this approved protocol will be documented, signed by the Study Director, dated, and maintained with this protocol. The Sponsor will be notified of any change or revision.

IX. RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocols, and final reports generated as a result of this study will be archived.

STUDY PROTOCOL - 3

CHROMOSOME ABERRATIONS ASSAY IN HUMAN WHOLE BLOOD LYMPHOCYTES WITH A CONFIRMATORY TRIAL USING MULTIPLE HARVESTS

I. OBJECTIVE

The objective of this *in vitro* assay is to evaluate the ability of a test article to induce chromosomal aberrations in human lymphocytes.

II. DEFINITION

Structural changes and rearrangements result from damage to the genetic material, and can be analyzed in cultured cells. Chromosomal breaks are mutations; many carcinogens cause chromosomal damage and chromosomal changes have been associated with cancer. Chromosomal aberrations are therefore a relevant test for potential mutagens and carcinogens.

- Chromosomal aberration: damage expressed in both sister chromatids at the same locus, or the exchange between both chromatids or more than one chromosome

- Chromatid aberration: damage seen in a single chromatid, or exchange between chromatids

III. RATIONALE

The assay aims to establish whether the test article can interact with cells to induce chromosomal breaks. Induced lesions may result in breaks in chromatin that are either repaired by the cell in such a way as to be undetectable or result in visible damage. Aberrations are a consequence of failure or mistakes in repair processes that result in breaks not rejoining, or rejoining in abnormal configurations (reviewed by Evans, 1962).

In order to detect aberrations we must obtain cells that are dividing (in metaphase) so that chromosomes are visible. The lymphocytes in blood do not usually divide; they are stimulated to divide in culture by phytohemagglutinin (PHA) and later arrested at metaphase by Colcemid®. Aberrations are examined when cells enter mitosis for the first time after chemical exposure, before they can be lost during the division process or be converted into complex derivatives during subsequent cell cycles. In the case of human lymphocytes, most dividing cells examined at about 48 hours of incubation are in their first mitosis (M1 cells).

IV. MATERIALS

A. Culture Method

Human venous blood will be collected from a healthy donor. Whole blood cultures will be initiated by adding 0.3 ml or 0.6 ml of fresh heparinized blood to 4.7 ml or 9.4 ml of culture medium, respectively, in each 15 ml centrifuge tube.

B. Culture Conditions

Cultures will be incubated with loose caps at about 37°C ± 2°C in a humidified incubator, in an atmosphere of about 5% ± 1.5% CO_2 in air. The medium will be RPMI 1640 supplemented with approximately 15% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and 1% PHA. The total culture volume will be 10 ml for the aberrations assay and 5 ml for the preliminary test for toxicity.

C. Test Article

The test article will be radio frequencyradiation at 837 MHz generated as specified by the Sponsor.

D. Control Articles

1. Negative controls

Negative controls will be cultures which contain only cells and culture medium.

2. Positive controls

Known mutagenic and chromosome breaking agents will be used at two or more concentrations to ensure adequate results, but only one concentration will be chosen for aberration analysis. Miitomycin C (MMC), a known clastogen, will be dissolved in water and used at concentrations of 25 to 300 ng/ml.

V. EXPERIMENTAL DESIGN

A. Treatment

The maximum exposure will be determined by the Sponsor, taking into account any relevant cytotoxicity information available.

B. Preliminary Test for Mitotic Suppression and Exposure Level Determination

Single 5 ml cultures with 0.3 ml whole blood will be set up. Single cultures will be initiated for each treatment (wide range of exposure levels and one negative control).

Approximately 48 hours after culture initiation, the cultures will be exposed and incubation will be continued at about 37°C for about 19 hours. At this time, Colcemid® (0.1 µg/ml, final concentration) will be added and incubation will be continued for an additional 1 to 2 hours. Total culture time will be 69 to 71 hours.

Then the cell suspension will be centrifuged, supernatant discarded, and cells treated with hypotonic KCl (0.075M) for up to 10 minutes to swell them. After centrifugation and removal of KCl, cells will be fixed and dropped onto slides to air dry. Slides will be stained with 5% Giemsa for 3 to 10 minutes and air dried.

Mitotic index will be analyzed from the top three (or more exposure levels if signs of severe toxicity are found) exposure levels by analyzing 1000 cells. Based on this information, exposure levels for the aberrations assays will be selected to ensure that there is ~50% suppression in the mitotic index at the highest exposure selected, unless no toxicity is observed at the highest exposure level specified by the Sponsor.

C. Treatment of Cultures with the Test Article for the Aberrations Assay

At least three exposure levels of the test article will be tested. Duplicate cultures will be used for the test article treatments, and negative controls, and single cultures will be used for each dose of the positive control.

Whole blood will be added to culture medium containing PHA and incubated at 37°C for about 48 hours. At this time the cells are beginning to divide due to the stimulation by PHA. Exposures will then be initiated and the cultures will be reincubated for a further ~19 hours. At this time, Colcemid® (0.1 µg/ml, final concentration) will be added and the cultures incubated for 1 to 2 hours more.

D. Lymphocyte Fixation

The cell suspension will be centrifuged, supernatant discarded, and cells treated with hypotonic KCl (0.075M) for up to 10 minutes to swell the cells. After centrifugation and removal of the KCl, cells will be washed three times with fixative (methanol:glacial acetic acid, 3:1) and dropped onto slides to air dry.

E. Cell Staining and Scoring

Slides will be stained with Giemsa and air dried. After drying, slides will be rinsed in xylene and mounted permanently. For control of bias, slides will be coded for analysis except for the positive controls, which will be checked first to make sure the aberration frequency is adequate. Cells will be selected for analysis on the basis of good morphology, and only cells with the number of centromeres equal to the modal number 46 are scored.

In order to select exposure levels for analysis, mitotic index will be analyzed first. The group selected for the highest exposure level will be that which suppressed mitotic index by ~50%. If no cellular toxicity is evident, the top three exposure levels tested will be analyzed. Two hundred cells

per exposure level (100 from each of the duplicate flasks) will be read from each of the top three exposure levels at which metaphase cells are available. Two hundred cells will be read from the negative controls. At least twenty-five cells will be read from one positive control and from those cultures that have greater than 50% of cells with one or more aberrations. (The complete number of 100 cells per culture may not be available due to toxicity or quality of preparations. The test will not, however, be considered adequate if less than 200 cells are available in the negative controls.) Percent polyploidy will be analyzed and tabulated. Mitotic index will also be tabulated.

Standard forms are used to record analyses. For each cell bearing an aberration, the microscope stage location is noted so the cell may be relocated if necessary. The complete list of aberrations scored and their definitions are attached to this protocol (pages B24-B26).

F. Confirmatory Assay

A confirmatory assay will be conducted using the same harvest time as the initial assay and a later harvest time of ~46 hours. Positive control cultures will be used only for the earlier harvest, unless the cultures for the two harvest times were initiated at different times. Cells will be cultured as in Section V. C., using the same procedure as in the regular harvest. Cell harvest and analysis of results will be done as in Sections V. D. and V. E.

TABLE 1 SUMMARY OF CHROMOSOMAL ABERRATIONS ASSAY TREATMENT SCHEDULE IN HOURS

Test	Test Article	Wash	Colcemid®	Fixation
Initial Assay	0	~19	~20.0	~22
Confirmatory Assay	0	~10	~20.0	~22
	0	~43	~44.0	~46

VI. DATA

A. Data Presentation and Evaluation

Data will be summarized in tables showing the numbers of cells analyzed, the types of aberrations found, frequencies of aberrations per cell, and percentages of cells bearing aberrations. Chromatid and isochromatid gaps will be noted but will not be added into the totals for aberration assessment since they may not be true breaks.

B. Assay Acceptance Criteria

An assay will be considered accepted for evaluation of test results only if all of the following criteria are satisfied:

- Unsatisfactory Controls

The assay will be repeated if:

i) The negative control is more than twice the upper limit of the range of historical control values (upper limit ~5% or more of cells with aberrations).

ii) The positive control result is not significantly higher ($p<0.01$) than the negative controls.

- Lack of Toxicity

If the aberration results are negative and there is no reduction in the mitotic index, the test must include the maximum exposure specified by the Sponsor.

- Excess Toxicity

The assay will be repeated if results are not available for at least three exposure levels.

- Sporadic Increase

If a significant increase is seen at one or more exposure levels but not in consecutive treatments, and if there is no clear evidence for a positive dose response, the assay will be repeated to verify the significance.

C. Assay Evaluation

The following factors will be taken into account in evaluation of the test article data:

- Overall aberration frequencies
- Percentage of cells with one aberration
- Percentage of cells with more than one aberration
- Any evidence for increasing amounts of damage with increasing exposure, (i.e., a positive dose response)

Statistical analysis will employ the Fishers Exact Test (Sokal and Rohlf, 1981) to compare the percentage of cells with aberrations in treated cells with results from the negative controls. A linear trend test of increasing number of cells with aberrations with increasing exposure levels (Armitage, 1971) will also be performed. The difference is considered significant when $p < 0.01$. The final evaluation of the test article will be based upon scientific judgment.